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A Novel *cis*-Regulatory Element, the PTS, Mediates an Anti-Insulator Activity in the *Drosophila* Embryo

Jumin Zhou and Michael Levine*
Department of Molecular Cell Biology
Division of Genetics and Development
University of California
Berkeley, California 94720

Summary

The *Abd-B Hox* gene contains an extended 3′ *cis*-regulatory region that is subdivided into a series of separate *iab* domains. The *iab-7* domain activates *Abd-B* in parasegment 12 (ps12), whereas *iab-8* controls expression in ps13. *iab-7* is flanked by two insulators, Fab-7 and Fab-8, which are thought to prevent regulatory factors, such as Polycomb silencers, from influencing neighboring *iab* domains. This organization poses a potential paradox, since insulator DNAs can work in a dominant fashion to block enhancer–promoter interactions over long distances. Here, we present evidence for a novel *cis*-regulatory sequence located within *iab-7*, the promoter targeting sequence (PTS), which permits distal enhancers to overcome the blocking effects of Fab-8 and the heterologous *su(Hw)* insulator. We propose that the PTS converts dominant, long-range insulators into local regulatory elements that separate neighboring *iab* domains.

Introduction

Since the discovery of eukaryotic enhancers 18 years ago (Banerji et al., 1981), just two additional classes of *cis*-regulatory sequences have been identified: silencer elements (Brand et al., 1985) and insulator DNAs (Kellum and Schedl, 1991). Silencers were first implicated in the regulation of yeast mating type loci. More recent studies have shown that localized patterns of *Hox* gene expression depend on *polycomb* response elements (PREs), which are thought to function like the prototypic yeast silencer and propagate long-range changes in chromatin structure (Pirrotta, 1997, 1998; Paro et al., 1998).

Insulator DNAs organize neighboring genes into separate chromatin domains (Udvardy et al., 1985; Kellum and Schedl, 1991, 1992; reviewed by Corces, 1995; Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Udvardy, 1999). The first insulators that were identified, *scs* and *scs′*, flank the *hsp70* locus and prevent heat shock regulatory elements from activating neighboring genes (Udvardy et al., 1985; Kellum and Schedl, 1991, 1992). Additional insulator DNAs have been subsequently identified, including the *suppressor of Hairy wing* [*su(Hw)*] insulator within the *gypsy* retrotransposon (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Dorsett, 1993), the chicken *globin* insulator (Chung et al., 1993, 1997; Bell et al., 1999), and the Fab-7 (*Frontabdominal-7*) and Fab-8 insulators within the bithorax complex (BX-C; Gyurkovics et al., 1990; Galloni et al., 1993; Karch et al., 1994; Hagstrom et al., 1996; Zhou et al., 1996, 1999; Mihaly et al., 1997, 1998; Barges et al., 2000). All of these insulators appear to function in a similar manner. They block gene expression only when positioned between a distal enhancer and target promoter. There are no obvious constraints on the range of this enhancer blocking activity (reviewed by Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Udvardy, 1999). For example, the *su(Hw)* insulator blocks the interaction of the distal *wing* enhancer in the *cut* locus (located nearly 90 kb 5′ of the gene) regardless of where *gypsy* inserts between the enhancer and promoter (Dorsett, 1993).

Abdominal-B (*Abd-B*) is regulated by an extended 3′ *cis*-regulatory region that encompasses approximately 50 kb of genomic DNA and contains all three classes of known *cis* elements: enhancers, silencers, and insulators (Busturia and Bienz, 1993; Hagstrom et al., 1996, 1997; Zhou et al., 1996, 1999; Mihaly et al., 1997; summarized in Figure 1). This 3′ regulatory DNA contains a series of *infra-abdominal* (*iab*) regions that control *Abd-B* expression in the fifth through eighth abdominal segments (parasegments 10–13; Lewis, 1978; Karch et al., 1985; Celniker et al., 1990; Boulet et al., 1991; Sanchez-Herrero, 1991; reviewed by Duncan, 1987). For example, *iab-5 cis*-regulatory elements control *Abd-B* expression in parasegment (ps) 10, while *iab-7* regulates expression in ps12. Each *iab* domain appears to contain at least one enhancer that initiates *Abd-B* expression in the early embryo, as well as a PRE silencer element that maintains the expression pattern throughout development (Busturia and Bienz, 1993; McCall et al., 1994; Hagstrom et al., 1996, 1997; Zhou et al., 1996, 1999; Busturia et al., 1997; Mihaly et al., 1997, 1998; Barges et al., 2000). It has been proposed that insulators flank each *iab* region and organize the *Abd-B* regulatory DNA into a series of separate chromatin loop domains (Gyurkovics et al., 1990; Galloni et al., 1993; reviewed by Vazquez et al., 1993; Mihaly et al., 1998). Consistent with this model is the recent demonstration that *iab-7* is flanked by two insulators, Fab-7 and Fab-8 (Zhou et al., 1999; Barges et al., 2000; summarized in Figure 1).

The occurrence of insulators in the *Abd-B* regulatory region raises a potential paradox, namely, how do distal enhancers such as IAB5 and IAB7 overcome Fab-7 and Fab-8 and interact with the *Abd-B* promoter? As discussed above, insulators are dominant and can block enhancer–promoter interactions even when located far from either the enhancer or target promoter. In this study, we report evidence for a novel *cis*-regulatory element, the promoter targeting sequence (PTS), which allows distal enhancers to overcome the blocking activities of insulator DNAs.

An unusual transvection process governs *Abd-B* regulation, whereby distant enhancers continue to interact with the *Abd-B* promoter even when translocated onto different chromosomes (Hendrickson and Sakonju, 1995;

*To whom correspondence should be addressed (e-mail: mlevine@uclink4.berkeley.edu).

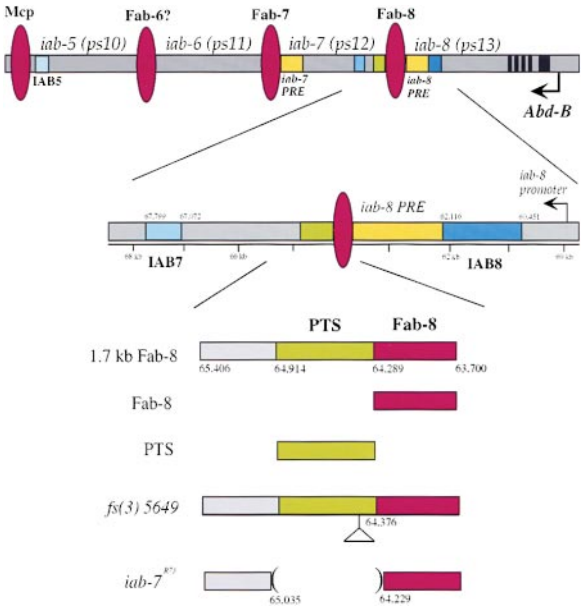

Figure 1. Summary of the *Abd-B* Locus

The *Abd-B* locus includes ~50 kb of 3′ *cis*-regulatory DNA (top). The leftward arrow indicates the *Abd-B* transcription start site, and the black rectangles correspond to exons present in the mature mRNA. Genetic studies suggest that *iab-8 cis*-regulatory elements are located both upstream and downstream of the *Abd-B* transcription unit; *iab-8* directs expression in parasegment (ps) 13. The remaining *iab* regulatory domains (*iab-5*, *iab-6*, and *iab-7*) map 3′ of the gene and activate *Abd-B* expression in ps10, ps11, and ps12, respectively. Recent studies suggest that each *iab* domain is flanked by insulator DNAs (indicated by the red ovals). Definitive evidence has been obtained only for the *Fab-7* and *Fab-8* insulators, which flank *iab-7*. A detailed map of the ~10 kb *transvection mediating region* (*tmr*) is shown below the *Abd-B* summary. The *tmr* has been shown to contain five *cis*-regulatory elements, including two enhancers, IAB7 and IAB8, the *Fab-8* insulator DNA, a PRE within *iab-8*, and an internal promoter that transcribes all of these elements in ps14 (see Zhou et al., 1999). This study provides evidence that the 1.7 kb *Fab-8* region contains two closely linked *cis*-regulatory elements, a minimal, 590 bp Fab-8 insulator, and a 625 bp promoter targeting sequence (PTS). The limits of the latter elements are shown below the 1.7 kb Fab-8 summary. Two previously identified mutations map within the PTS, including a *P* element insertion [*fs(3)5649* or *P302*] and a small deletion (*iab-7*^{R73}). This latter mutation is also called *Fab-7 R73* and contains a second deletion in the Fab-7 insulator (Gyurkovics et al., 1990; Hendrickson and Sakonju, 1995). Sequence analysis (data not shown) indicates that the *iab-7*^{R73} deletion is 806 bp in length and removes the entire PTS.

Hopmann et al., 1995; Sipos et al., 1998). Transvection depends on an ~10 kb region located just downstream of the *Abd-B* transcription unit called the *transvection mediating region* (*tmr*) (Hopmann et al., 1995; Sipos et al., 1998, summarized in Figure 1). The *tmr* contains two embryonic enhancers, IAB7 and IAB8, as well as the Fab-8 insulator (Zhou et al., 1999).

In the present study, we conduct additional analyses of the *tmr* in an effort to identify regulatory sequences that might mediate long-range enhancer–promoter interactions. The 1.7 kb Fab-8 region is shown to contain two closely linked elements: an insulator and the PTS. In transgenic assays, the PTS permits distal enhancers to overcome the blocking effects of Fab-8 and the heterologous *su(Hw)* insulator. We suggest that the PTS is responsible, at least in part, for converting dominant, long-range insulators into local regulatory elements that isolate neighboring *iab* domains.

Results

Identification of the Minimal Fab-8 Insulator

Previous studies mapped Fab-8 within a 1.7 kb genomic DNA fragment in the *tmr* (Zhou et al., 1999; Barges et al., 2000; summarized in Figure 1). To identify the minimal insulator, smaller Fab-8 DNA fragments were placed between two test enhancers, H1 and IAB5, that activate two divergently transcribed reporter genes, *white* and *lacZ* (Figure 2; data not shown). These reporter genes can be independently assayed by in situ hybridization using either a digoxigenin-labeled *white* antisense RNA probe or a digoxigenin-labeled *lacZ* probe. A neutral DNA spacer sequence permits both enhancers to activate the leftward *white* reporter gene (Figure 2A) and rightward *lacZ* gene (Figure 2B). Both reporter genes exhibit composite staining patterns consisting of anterior (H1) and posterior (IAB5) bands of expression. The replacement of the spacer sequence with a minimal, 590 bp Fab-8 fragment leads to a selective block in IAB5–*white* and H1–*lacZ* interactions (Figures 2C and 2D; see diagrams below the embryos). Thus, *white* is activated only by the proximal H1 enhancer, while *lacZ* is activated by IAB5. The minimal insulator is located in the proximal portion of the original 1.7 kb Fab-8 sequence (summarized in Figure 1).

Genetic studies provide additional support for this mapping of Fab-8 (Figures 2E and 2F). A previously identified enhancer trap line, *fs(3)5649* or *P302* (Castrillon et al., 1993), is inserted within the *iab-7* domain, immediately adjacent to the minimal Fab-8 insulator (Figures 2E and 2F; see diagrams). In situ hybridization was used to examine the expression of the *lacZ* reporter gene contained within the inserted *P* element (Figure 2E). Strong expression is detected in ps12, weaker staining is seen in ps14, and there is little or no staining in ps13. This result indicates that *P302* is activated by *cis* elements contained within *iab-7*. The absence of staining in ps13 suggests that *iab-8* elements fail to activate *lacZ*. As shown previously, the IAB8 enhancer directs expression in ps13 and ps14 (Figure 2F), whereas IAB7 mediates expression in ps12 and ps14 (Zhou et al., 1999). The distinct *P302* (Figure 2E) and IAB8/*lacZ* (Figure 2F) staining patterns suggest that Fab-8 blocks IAB8–*P302* interactions.

The *tmr* Contains a Promoter Targeting Activity

The minimal, 590 bp Fab-8 insulator can block the distal *rhomboid* NEE when positioned 3′ of the *lacZ* reporter gene (Figure 3). In these experiments the H1 enhancer was placed 5′ of the *white* and *lacZ* reporter genes as an internal control (Figures 3A and 3B; see diagrams). In the absence of Fab-8, both enhancers mediate composite patterns of *white* and *lacZ* expression that consist of an anterior band (H1) and lateral stripes (NEE). The insertion of Fab-8 between *lacZ* and the 3′ NEE alters the *white* and *lacZ* staining patterns (Figures 3C and 3D). Expression is now detected only in anterior regions,

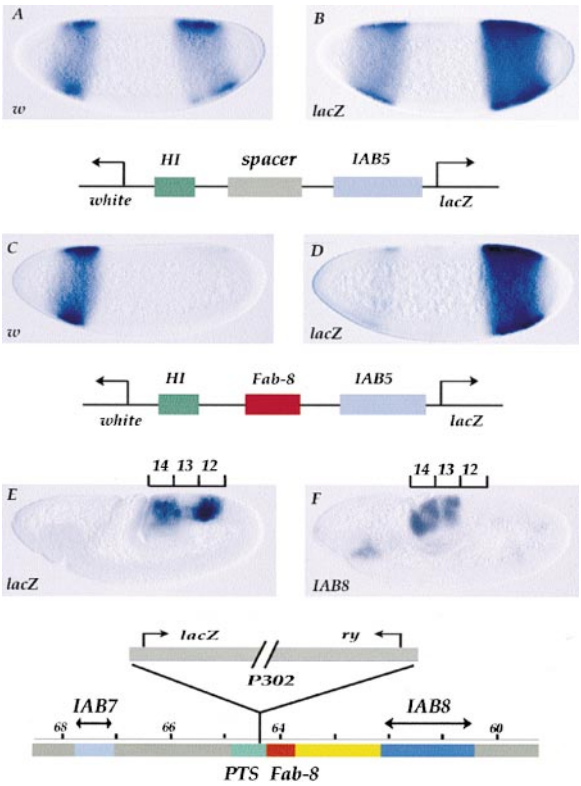

Figure 2. Identification of the Minimal *Fab-8* Insulator

(A–D) Transgenic embryos carrying the indicated *P* elements were hybridized with either *white* (A and C) or *lacZ* antisense RNA probes. The *hairy* H1 enhancer and IAB5 enhancer were placed 5′ of the two reporter genes. The two enhancers are separated by either a spacer sequence (A and B) or *Fab-8* DNA (C and D).

(A) *white* expression pattern. Staining is detected in both anterior (H1) and posterior (IAB5) regions, indicating that both enhancers activate the *white* reporter gene.

(B) Same as (A) except that the embryo was stained with a *lacZ* probe. Staining is detected in both anterior and posterior regions, although the proximal IAB5 enhancer directs somewhat stronger expression than the distal H1 enhancer.

(C) *white* staining pattern. The replacement of the spacer sequence with a 590 bp *Fab-8* DNA alters the expression pattern (compare with [A]). Staining is restricted to anterior regions, indicating that the proximal H1 enhancer activates *white*, whereas the distal IAB5 enhancer is blocked.

(D) *lacZ* staining pattern. The *Fab-8* DNA attenuates H1–*lacZ* interactions, so that the reporter gene exhibits only weak expression in anterior regions. In contrast, the proximal IAB5 enhancer continues to mediate intense staining.

(E) *lacZ* pattern in an elongating embryo carrying the previously identified *P302 P* element insertion (Castrillon et al., 1993), which maps just 87 bp from the the minimal, 590 bp *Fab-8* insulator within *iab-7*. The *lacZ* reporter gene is expressed strongly in ps12 and weakly in ps14, indicating activation by *iab-7* elements such as the IAB7 embryonic enhancer. The absence of staining in ps13 suggests that *iab-8 cis*-regulatory elements are unable to activate the reporter gene. This block is presumably due to the *Fab-8* insulator.

(F) A transgenic embryo that contains the 1.6 kb IAB8 enhancer linked to a *lacZ* reporter gene (see Figure 4B). Staining is detected in ps13 and ps14. This pattern is distinct from the one shown in (E).

indicating that the NEE is blocked but the 5′ H1 enhancer continues to activate both reporter genes.

Unexpected results were obtained when the "full-length" 9.5 kb *tmr* was placed between *lacZ* and the 3′

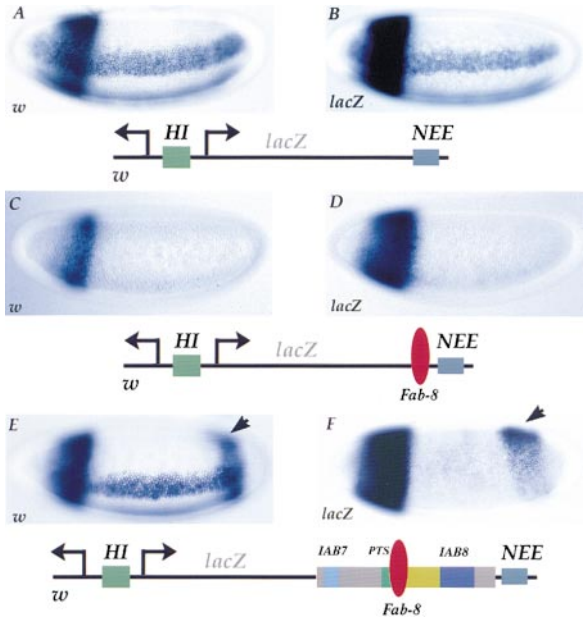

Figure 3. The *tmr* Contains a Promoter Targeting Activity

Transgenic embryos express the indicated *white* and *lacZ* reporter genes. Embryos are at the midpoint of nuclear cleavage cycle 14 and are oriented with anterior to the left and dorsal up.

(A and B) *white* (*w*) and *lacZ* staining patterns, respectively, obtained with fusion genes that contain the *hairy* H1 enhancer located 5′ of the reporter genes, and the *rhomboid* NEE located 3′ of *lacZ*. Both reporter genes exhibit composite staining patterns consisting of an anterior band (H1) and a lateral stripe (NEE). In these lateral views, only one of the two symmetric lateral stripes can be detected in the presumptive neurogenic ectoderm. The other lateral stripe is out of the focal plane of the photos.

(C and D) Same as (A) and (B) except that the minimal 590 bp Fab-8 insulator was placed between the 3′ end of the *lacZ* transcription unit and the distal NEE. The insulator blocks the NEE, so that neither *white* nor *lacZ* exhibits lateral stripes of expression. In contrast, the 5′ H1 enhancer continues to activate both reporter genes in anterior regions.

(E and F) Same as (A)–(D) except that the full-length, 9.5 kb *tmr* was placed between *lacZ* and the 3′ NEE. The locations of the previously identified *cis*-regulatory elements are indicated in the diagram; the Fab-8 insulator (red oval) is located near the center of the *tmr*. The distal *white* reporter gene is activated by the 3′ NEE and is expressed in lateral neurogenic stripes. The band of *white* expression in anterior regions is due to the 5′ H1 enhancer. There is also a posterior stripe of staining (arrow) resulting from the native IAB8 enhancer contained within the *tmr*. In more dorsal views, this posterior staining pattern sometimes extends to more anterior regions, indicating weak activation by the IAB7 enhancer (data not shown). The *lacZ* reporter gene is not activated by the 3′ NEE but is instead activated by the control 5′ H1 enhancer and the IAB7 enhancer (arrowhead) contained within the *tmr*. About half the transgenic lines carrying this fusion gene exhibit the staining patterns presented in this figure. The other half show a composite pattern of *lacZ* expression that includes NEE lateral stripes (data not shown).

NEE (Figures 3E and 3F). Instead of observing a simple block in NEE activity, *white* and *lacZ* exhibited variable activation by the 3′ NEE despite the presence of the Fab-8 insulator within the *tmr* (Figures 3E and 3F; see diagram). Four out of nine transgenic lines carrying this fusion gene exhibit selective NEE–*white* interactions, so that only the distal *white* reporter gene exhibits lateral stripes of expression (Figure 3E). The proximal *lacZ* gene

is not activated by the NEE but continues to exhibit expression in anterior regions, indicating activation by the control 5′ H1 enhancer (Figure 3F). In contrast, all transgenic lines carrying the parental H1-NEE fusion gene exhibit lateral stripes of both *white* and *lacZ* expression (e.g., Figures 3A and 3B).

These results suggest that the *tmr* contains two regulatory activities, possibly mediated by the same *cis* element. It permits the distal NEE to overcome the Fab-8 insulator and restricts expression to the *white* reporter gene. Further evidence for this latter activity stems from the observation that the IAB7 enhancer contained within the *tmr* primarily activates the *lacZ* reporter gene (arrowhead, Figure 3F), while IAB8 activates *white* (arrow, Figure 3E). We refer to this activity as "promoter targeting." A distal enhancer mainly interacts with one, but not both, target genes.

In an effort to identify the promoter targeting sequences within the *tmr*, we analyzed the activities of a minimal, 4 kb *tmr* that contains the 1.7 kb Fab-8 region positioned between minimal IAB7 and IAB8 enhancers (Figures 4C and 4D; see diagrams). A minimal, 1.6 kb IAB8 enhancer directs a posterior band of *lacZ* expression in ps13 and ps14 (Figure 4B), whereas a modified 700 bp IAB7 enhancer (IAB7*) activates the *white* reporter gene in ps12, ps14, and the presumptive thorax (Figure 4A). This latter staining is due to the removal of critical Krüppel repressor sites (Zhou et al., 1999). The 1.7 kb Fab-8 DNA was inserted between the IAB8 and IAB7* enhancers, downstream of *lacZ* (Figure 4, see diagram below [C] and [D]).

The distal IAB7* enhancer selectively activates the *white* reporter gene, as judged by the distinctive features of the staining pattern including two posterior stripes and staining in the presumptive thorax (Figure 4C; compare with [A]). IAB7* does not activate *lacZ*. In contrast, the proximal IAB8 enhancer mainly activates *lacZ* (Figure 4D; compare with 4B), but might also weakly activate *white* (Figure 4C). In the absence of the 1.7 kb Fab-8 DNA, the distal IAB7* enhancer fails to activate either *white* or *lacZ* (Figures 4E and 4F). Both reporter genes are activated solely by IAB8.

The promoter targeting and anti-insulator activities of the 1.7 kb Fab-8 fragment were confirmed by the use of the Flp-*frt* system (Golic and Lindquist, 1989). The 3′ Fab-8 DNA was flanked by *frt*-binding sites (indicated by rightward arrows in the diagram beneath Figure 4D). The *P* transformation vector was introduced into males that express the Flp recombinase under the control of a sperm-specific *tubulin* promoter (Wu et al., 1998). The recombinase binds the *frt* sites and deletes the Fab-8 DNA (see diagram beneath Figure 4F). A total of four independent transgenic lines were analyzed before and after deletion of Fab-8. In every case, the removal of Fab-8 results in the loss of IAB7–*white* interactions. These results suggest that the activation of *white* is not due to random position effects, but is specifically mediated by promoter targeting sequences contained within the 1.7 kb Fab-8 DNA. The most important implication of this experiment is that the 1.7 kb fragment contains sequences that permit the distal IAB7* enhancer to overcome the blocking activity of the minimal Fab-8 insulator.

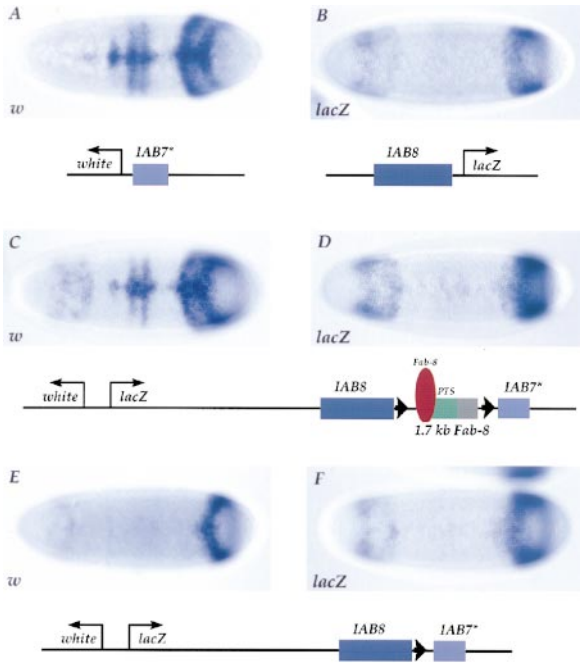

Figure 4. The 1.7 kb Fab-8 DNA Facilitates Enhancer–Promoter Interactions

Dorsal views of gastrulating transgenic embryos that express the indicated *P* elements after hybridization with *white* or *lacZ* RNA probes. The 1.7 kb *Fab-8* DNA was inserted between IAB8 and a defective IAB7 enhancer (IAB7*) located 3′ of *lacZ* (see diagrams below [C] and [D]).

(A) *white* staining pattern obtained with a 700 bp IAB7 enhancer that lacks Kr repressor sites. Expression is detected in two posterior stripes, ps12 and ps14, as well as anterior half-stripes in the presumptive thorax. The latter pattern is due to the loss of the Kr sites.

(B) *lacZ* staining pattern obtained with the IAB8 enhancer. Expression is detected in a broad posterior band that includes ps13 and ps14. Weaker staining is also detected in anterior regions (presumptive head).

(C and D) *white* and *lacZ* staining patterns obtained with the "minimal *tmr*" positioned 3′ of *lacZ*. This minimal, 4 kb *tmr* contains the IAB8 and IAB7* enhancers, as well as the 1.7 kb Fab-8 DNA placed between the two enhancers (see diagram). The *white* reporter gene exhibits nearly the same pattern as that obtained with the IAB7* enhancer (A). There are two posterior stripes, as well as anterior half-stripes in the thorax. This pattern indicates that the 3′ IAB7* enhancer can overcome the Fab-8 insulator within the minimal *tmr* and activate *white*. However, the posterior-most ps14 stripe is relatively stronger in this embryo than in (A). In addition, there is weak staining in head regions. These observations suggest that the IAB8 enhancer can weakly activate *white*. The *lacZ* reporter gene appears to be activated solely by IAB8, since staining is restricted to posterior and head regions (D). The absence of staining in the thorax suggests that IAB7* fails to activate *lacZ*.

(E and F) *white* and *lacZ* staining patterns in the same transgenic line as shown in (C) and (D). The *Fab-8* DNA is flanked by *frt* sites (see diagram under [D]) and was deleted by introducing the transgene into males expressing the Flp recombinase under the control of a sperm-specific *tubulin* promoter. Both reporter genes are activated solely by IAB8. The distal IAB7* enhancer is inactive, possibly due to short-range repressors bound to IAB8.

Identification of the Minimal PTS

A transgenic embryo assay was devised to identify the minimal sequences within the *tmr* and 1.7 kb Fab-8 DNA that abrogate the enhancer blocking activity of the Fab-8 insulator. Particular efforts focused on a 625 bp fragment that contains the *P302* insertion (see Figure 2) and

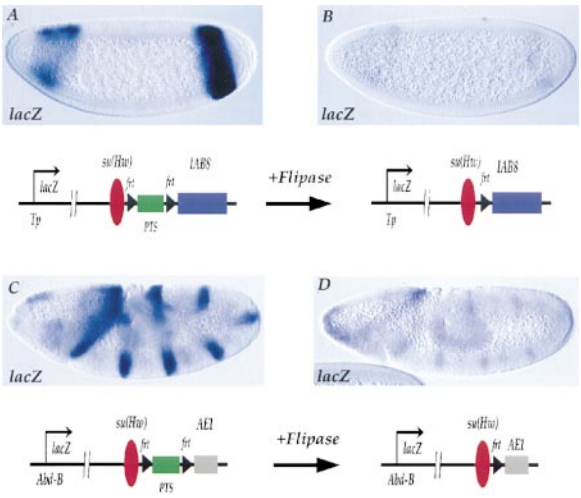

Figure 5. The 625 bp PTS Mediates an Anti-Insulator Activity

Transgenic embryos carrying the indicated *P* elements were hybridized with a *lacZ* antisense RNA probe. Cellularizing embryos are shown in (A) and (B), whereas the embryos in (C) and (D) are undergoing germband elongation.

(A) The 1.6 kb IAB8 enhancer was placed downstream of a 360 bp *su(Hw)* insulator from the *gypsy* retrotransposon (see diagram). The 625 bp PTS was inserted between the insulator and 3′ IAB8 enhancer (note that the PTS is flanked by minimal *frt* recombination elements). Intense expression of the *lacZ* reporter gene is detected in posterior regions (ps13 and ps14). Weaker staining is also detected in the presumptive head, suggesting that the minimal IAB8 enhancer might lack critical repressor elements.

(B) Same as (A) except that the PTS was deleted via Flp-mediated recombination. The *lacZ* reporter gene is essentially inactive, indicating that the *su(Hw)* insulator blocks the 3′ enhancer in the absence of the PTS.

(C) A 480 bp AE1 enhancer was placed downstream of the *su(Hw)* insulator. The PTS was inserted between the insulator and 3′ enhancer. Intense stripes of *lacZ* expression are detected, indicating that the AE1 enhancer is able to "jump over" the *su(Hw)* insulator and activate the reporter gene.

(D) Same as (C) except that the PTS was deleted via Flip-mediated recombination. The *lacZ* reporter gene is virtually silent, indicating that the *su(Hw)* insulator blocks the 3′ AE1 enhancer.

is adjacent to the mimimal Fab-8 insulator (summarized in Figure 1). This fragment does not possess an enhancer blocking activity, even when placed in a 5′ position (data not shown; see blocking assay in Figure 2). However, when positioned 3′ of *lacZ*, the 625 bp PTS is able to suppress the enhancer blocking activities of the *su*(*Hw*) insulator from the *gypsy* retrotransposon (Figure 5).

Two different distal enhancers were tested in these assays, the minimal IAB8 enhancer (Figures 5A and 5B) and the heterologous *ftz* autoregulatory enhancer (AE1; Figures 5C and 5D; see Pick et al., 1990; Schier and Gehring, 1992). These enhancers were placed downstream of the *lacZ* reporter gene, over 5 kb from either the *Transposase* (*Tp*) promoter (Figures 5A and 5B) or a minimal *Abd-B* promoter sequence (Figures 5C and 5D). The 340 bp *su(Hw)* insulator was inserted between the 3′ end of *lacZ* and each distal enhancer. In the absence of the PTS, the insulator blocks both IAB8 (Figure 5B) and AE1 (Figure 5D). However, insertion of the PTS just 3′ of the insulator results in strong activation of *lacZ* by the IAB8 (Figure 5A) and AE1 (Figure 5C) enhancers.

AE1 directs seven stripes of *lacZ* expression in response to endogenous *ftz* products (Figure 5C). IAB8 directs both an intense posterior stripe of *lacZ* expression as well as weaker staining in anterior regions (Figure 5A). The minimal enhancer might lack repressor elements that normally block this anterior staining (see Figure 4). The Flp-*frt* strategy was used to eliminate complications in interpretation due to position effects (see Figure 4). Eight independent transgenic lines exhibited intense *lacZ* expression when the PTS was positioned between the insulator and 3′ IAB8 enhancer (represented by the staining shown in Figure 5A). In every case, *lacZ* expression was virtually lost upon removal of the PTS (e.g., Figure 5B). In a ninth transgenic line, IAB8 activated the distal *white* reporter gene rather than *lacZ*, and this expression persisted after removal of the PTS. These results suggest that the site of insertion interfered with the enhancer blocking activity of the *su(Hw)* insulator in just one of the nine lines tested.

Most of the transgenic lines carrying the 625 bp PTS exhibit selective expression of *lacZ* and not *white*. In contrast, in the absence of the PTS, the 3′ IAB8 enhancer activates both *lacZ* and *white* with nearly equal efficiency (e.g., Figures 4E and 4F). The selective activation of *lacZ* suggests that the minimal, 625 bp PTS mediates both promoter targeting and the anti-insulator activity.

Genetic Analysis

Genetic studies were done to determine whether PTS activity is important for enhancer–*Abd-B* interactions in the context of the BX-C (Figure 6). Two previously identified mutations disrupt the PTS, *fs(3)5649* (*P302*) and *iab-7*^{R73} (Gyurkovics et al., 1990; Castrillon et al., 1993). The former corresponds to a *P* element insertion (see Figure 2), while the latter is an 806 bp deletion that removes the entire minimal PTS (summarized in Figure 1). Each mutation produces viable adults when placed in *trans* over the *S10* deficiency [Df(3R)89B9-16; 89E3-4]. *S10* lacks 3′ *Abd-B cis*-regulatory elements, including *iab-5*, *iab-6*, *iab-7*, and the Fab-8 insulator and IAB8 enhancer (Karch et al., 1990). Neither mutation causes obvious adult cuticular phenotypes when placed in *trans* with a "wild-type" balancer chromosome (Figures 6B–6D; compare with Figure 6A). However, *S10/P302* and *S10/iab-7*^{R73} transheterozygotes exhibit abdominal transformations (Figures 6E and 6F).

The *P302* mutation produces a transformation of A7 to an A6/A5 segment identity, as well as a weaker A6 to A5 transformation (Figure 6E). These phenotypes are consistent with a reduction in IAB7–*Abd-B* interactions (Figure 6E). In addition, there is a slight transformation of A5 to A4, as judged by the pattern of pigmentation (arrow, Figure 6E) and the trichome pattern (data not shown). This phenotype is consistent with reduced IAB5–*Abd-B* interactions. Further evidence for reduced *iab-5* activity is provided by the more complete transformation of A5 to A4 obtained with the *iab-7*^{R73} deletion (see large arrow, Figure 6F). The *iab-7*^{R73} deletion also results in the transformation of A7 and A6 into A4/A5 segment identities. These results suggest that mutations in the PTS impair the interactions of distant enhancers, such as IAB5 and IAB7, with the *Abd-B* promoter (see below).

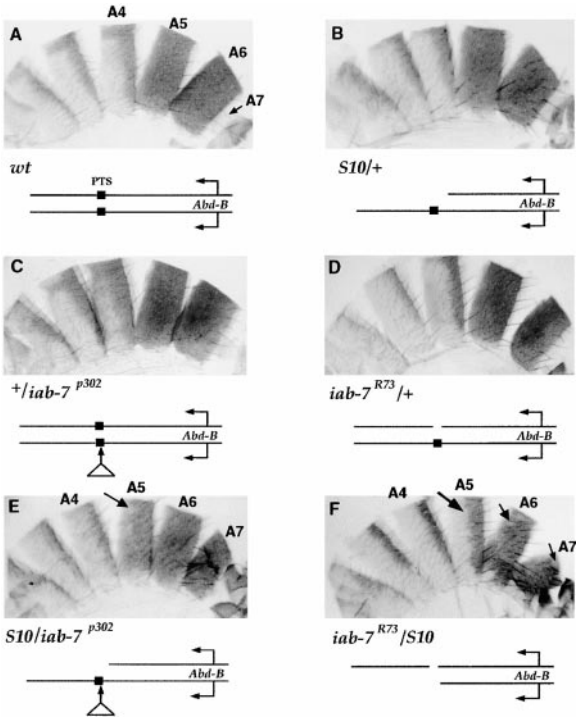

Figure 6. The PTS Is Required for Normal *Abd-B*$^{+}$ Gene Activity

Dorsal cuticles (tergites) were prepared from adult males of the indicated genotypes

(A) Oregon^{R}. Wild-type males exhibit pigmentation in posterior regions of the second, third, and fourth tergites. In contrast, the fifth and sixth tergites are fully pigmented, while the vestigial seventh tergite is barely discernible.

(B) Heterozygous male carrying the *S10* deletion *[Df(3R)89B9-16;89E3-4]* and a "wild-type" balancer chromosome *[Dp (3,3) P5, Sb*]. The *S10* defeciency removes all *Abd-B cis*-regulatory elements to the left of the internal *iab-8* promoter (see Figure 1), including the IAB8 enhancer, the Fab-8 insulator, the PTS, as well as the *iab-5*, *iab-6*, and *iab-7* domains. There are no obvious changes in the normal abdominal pigmentation pattern.

(C) Heterozygous male carrying the *P302 P* element insertion and the "wild-type" *CxD* balancer chromosome. A normal segmentation pattern is observed.

(D) Heterogyzous male carrying the *iab-7*^{R73} deficiency chromosome, which contains an 806 bp deletion that removes the PTS, and a "wild-type" chromosome (*TM6Tb*). A normal abdominal pigmentation pattern is observed.

(E) Heterozygous male carrying the *S10* deficiency chromosome and the *p302* insertion chromosome. The A7 tergite is now fully developed, indicating a transformation toward an A5/A6 identity (based on sternite pattern). In addition, the A5 tergite is partially transformed toward A4, as indicated by the partial loss of pigment in anterior regions (arrow). These transformations indicate a partial loss of *Abd-B*$^{+}$ activity.

(F) Heterozygous male carrying the *iab-7*^{R73} deletion and *S10* deficiency. The A7 segment is fully developed, and both A6 and A7 exhibit pigmentation patterns that resemble intermediates of the A4 and A5 patterns (small arrow). The fifth tergite exhibits the A4 pigmentation pattern (large arrow), indicated by the complete loss of pigmentation in the anterior half of the dorsal cuticle. These phenotypes are more severe than those shown in (E), suggesting that the removal of the PTS causes a substantial reduction in *Abd-B*$^{+}$ activity.

Discussion

Previous genetic studies identified a ~10 kb region 3′ of *Abd-B* that is essential for long-range enhancer–promoter interactions (Hendrickson and Sakonju, 1995; Hopmann et al., 1995). This regulatory region, the *tmr*, contains a dense assortment of *cis* elements, including an insulator DNA (Fab-8), two enhancers (IAB7 and IAB8), a PRE, and an internal promoter (Zhou et al., 1999; Barges et al., 2000). The present study provides evidence that the 1.7 kb Fab-8 insulator region contains two closely linked regulatory elements: the minimal 590 bp Fab-8 insulator, and a promoter targeting sequence (PTS), which abrogates the enhancer blocking activities of Fab-8 and the heterologous *su(Hw)* insulator. We suggest that the newly identified PTS modifies the regulatory properties of insulator DNAs and facilitates long-range enhancer–promoter interactions.

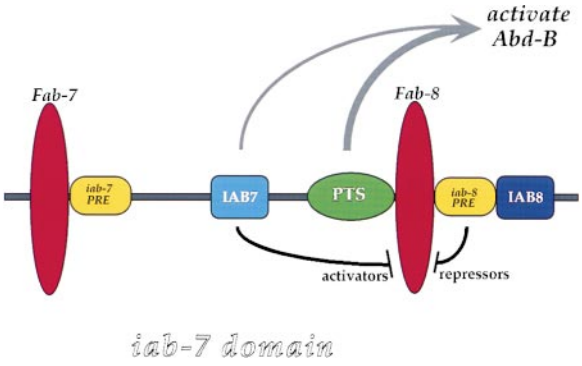

Figure 7. Model for PTS-Insulator Activities

The *iab-7* domain is flanked by the Fab-7 and Fab-8 insulators. Fab-8 prevents *iab-8* repressors from inactivating *iab-7* regulatory elements in ps12. It is conceivable that Fab-8 also blocks local interactions between IAB7 activators and *iab-8* elements, which could cause ectopic activation of IAB8 in ps12. The PTS permits distal enhancers, such as IAB7, to overcome the blocking effects of the Fab-8 insulator and activate the *Abd-B* promoter. Thus, the PTS converts the dominant, long-range Fab-8 insulator into a short-range element that prevents local cross-regulatory interactions between *cis* elements in the *iab-7* and *iab-8* domains.

Properties of the PTS

The full-length, 9.5 kb *tmr* and the 1.7 kb Fab-8 DNA mediate two unusual regulatory activities. They can target 3′ enhancers to the distal *white* reporter gene and suppress the enhancer blocking activity of the minimal Fab-8 insulator. Neither activity was detected when the 1.7 kb Fab-8 DNA was placed 5′ of test promoters (Zhou et al., 1999; e.g., see Figure 2). In the 5′ position, the DNA fragment exhibits a simple insulator activity, whereby distal, not proximal, enhancers are selectively prohibited from activating *white* and *lacZ* (Zhou et al., 1999). Additional regulatory activities were uncovered only when the *tmr* and 1.7 kb Fab-8 DNAs were placed far (>5 kb) from the target promoters.

The PTS suppresses the enhancer blocking activities of Fab-8 when positioned in either orientation relative to the insulator or target promoter (see Figures 3 and 5). There does not seem to be a requirement for tight linkage of the PTS and distal enhancer. For example, the *rho* NEE overcomes Fab-8 even when the PTS and NEE are separated by ~5 kb (see Figure 3). The PTS abrogates enhancer blocking when located either upstream or downstream of the insulator (see Figures 3 and

5). It is currently unclear whether anti-insulator activity depends on close proximity of the PTS and insulator.

We propose that proteins bound to the PTS somehow stabilize enhancer–promoter complexes and thereby help distal enhancers overcome the blocking activity of an intervening insulator. Moreover, a stable enhancer–promoter complex might prevent the enhancer from interacting with additional promoters once a target promoter is selected. PTS elements might play a role in the selection of a single receptor gene in mammalian odorant complexes (Mombaerts et al., 1996). An alternative model is that the PTS works in a local fashion to inhibit linked insulators. For example, proteins bound to the PTS might physically interact with proteins bound to neighboring insulators.

Role of the PTS in the BX-C

Genetic studies suggest that the PTS can facilitate enhancer–promoter interactions even when the distal enhancer is located far from both the PTS and target promoter. The *R73* (*iab-7*^{R73}) chromosome contains a deletion in the Fab-7 insulator as well as in the PTS (see Figure 1 summary). The loss of Fab-7 causes a dominant tranformation of A6 into A7 (Mihaly et al., 1997), suggesting that *iab-7* activators "spread" into the *iab-6* domain and result in the overexpression of *Abd-B* in A6 (ps11). The *R73* deletion suppresses this transformation (Gyurkovics et al., 1990), possibly due to a reduction in *iab-6–Abd-B* interactions resulting from the loss of the PTS. Moreover, *R73/S10* transheterozygotes exhibit a relatively robust homeotic transformation of A5 into A4 (Figure 6). This observation suggests that the loss of the PTS also reduces IAB5–*Abd-B* interactions; the IAB5 enhancer is located ~40 kb away from the PTS.

We propose that the PTS is responsible for converting dominant, long-range insulator DNAs into local, short-range regulatory elements (Figure 7). The Fab-7 and Fab-8 insulators prevent *iab-7* and *iab-8 cis*-regulatory elements from disrupting the activities of neighboring *iab* domains. For example, the IAB8 enhancer directs *Abd-B* expression in ps13 and may be essential for the morphogenesis of the eighth abdominal segment (Karch et al., 1985; Celniker et al., 1990; Boulet et al., 1991; Sanchez-Herrero, 1991; McCall et al., 1994; Zhou et al., 1999). IAB8 activity is inhibited in all anterior segments, extending through ps12, and the *iab-8* PRE maintains this pattern of repression throughout development (Zhou et al., 1999; Barges et al., 2000; see Figure 7). It is thought that Pc-G repressors bound to PREs work over long distances, possibly by propagating changes in chromatin structure (reviewed by Pirrotta, 1997, 1998; Paro et al., 1998). The Fab-8 insulator may be essential for preventing these repressors from spreading into *iab-7* and inactivating the IAB7 enhancer in ps12 (Mihaly et al., 1997; Mallin et al., 1998; Barges et al., 2000; reviewed by Mihaly et al., 1998). Similarly, Fab-8 might also prevent *iab-7* activators from inducing ectopic expression of IAB8 in ps12 (see Figure 7). However, like other insulators, Fab-7 and Fab-8 can block distal enhancers over long distances (e.g., Dorsett, 1993; Galloni et al., 1993; Zhou et al., 1996; see Figures 3C and 3D). The newly identified PTS appears to permit distal enhancers to overcome the blocking effects of Fab-7 and Fab-8, but might not interfere with the local isolation of neighboring *iab* domains.

Recent genetic studies suggest that transvection depends on both the *tmr* and regulatory sequences in the *Abd-B* 5′ promoter region (Sipos et al., 1998). It has been suggested that these 5′ regulatory elements help tether distal *iab* enhancers to the *Abd-B* promoter (Sipos et al., 1998). Thus, the PTS might work in concert with dedicated enhancer–promoter interactions to ensure that distant *iab* elements can overcome the blocking effects of intervening insulators. Specific enhancer–promoter interactions have been observed in a number of *Drosophila* loci, including the Antennapedia complex (Ohtsuki et al., 1998), *gooseberry* (Li and Noll, 1994), and *dpp* (Merli et al., 1996).

Experimental Procedures

Plasmid Constructions

The H1-spacer-IAB5 *P* element transformation vector shown in Figures 2A and 2B is described by Zhou et al. (1996). In Figures 2C and 2D, the 1.0 kb spacer sequence was replaced with the proximal 590 bp BamHI PCR fragment from the 1.7 kb Fab-8 DNA (see Figure 1).

The *P* transformation vector used in Figures 3A and 3B was prepared as follows. First, the pBluescript SK$^+$ vector was modified by converting the KpnI site into a BglII site. A 300 bp fragment of the NEE enhancer (Ip et al., 1992) was cloned into the EcoRI site of the modified vector. The NEE was then removed as a BglII-BamH1 fragment and inserted into the unique BglII site located at the 3′ end of the *lacZ* gene in the C4PLZ vector. A 230 bp fragment of the *hairy* stripe I enhancer (HI; Zhou et al., 1996) was inserted between the SpeI and BamHI sites 5′ of *lacZ*. The *P* element used in Figures 3C and 3D was prepared by inserting a 590 bp BamH1 Fab-8 insulator DNA into the unique BglII site located between *lacZ* and the 3′ *rho* NEE. Similarly, the P element used in Figures 3E and 3F was prepared by inserting the complete 9.5 kb *tmr* into the BglII site of the same vector. The *tmr* was previously cloned between the BamHI and HindIII sites of the modified pBluescript and then excised as a BamHI-BglII fragment.

The *P* element transformation vector used in Figure 4B was prepared with a 1.6 kb EcoRI-PstI fragment contained within the original 2.7 kb IAB8 enhancer described by Zhou et al. (1999). The fragment was cloned into the EcoRI-PstI sites of the modified pBluescript vector, and then a BamHI-BglII fragment containing the minimal IAB8 enhancer was cloned into the BamHI site of the C4PLZ *P* element vector 5′ of the *lacZ* reporter gene. To generate the transgene in Figure 4A, the defective 700 bp IAB7* enhancer containing mutations in Krüppel-binding sites (Zhou et al., 1999) was cloned into the SmaI-EcoRI sites of the modified pBluescript vector described above. A BamHI-BglII fragment containing IAB7 was inserted into the BamHI site of the C4PLZ vector.

The *P* element shown in Figures 4C and 4D was prepared by inserting the 1.6 kb BamHI-BglII IAB8 fragment into the 3′ BglII site of the C4PLZ vector. The 700 bp BamHI-BglII fragment of IAB7* was subsequently inserted into the recreated BglII site, generating C4PLZ/IAB8/IAB7* with a unique BglII site located between IAB7* and IAB8. The 1.7 kb Fab-8 DNA (Zhou et al., 1999) was first cloned into the unique BamHI site of a modified C4PLZ/>> vector containing *frt* (>) sites flanking BamHI. The >Fab-8> DNA was isolated as a BglII fragment and inserted into the BglII site of the C4PLZ/IAB8/IAB7* *P* element, thereby creating the final C4PLZ/IAB8>Fab-8>IAB7* transformation vector.

The transformation vector shown in Figure 5A was prepared by inserting a 360 bp BamHI-BglII fragment containing the *gypsy su(Hw)* insulator (Cai and Levine, 1995) into the BglII site of the C4PLZ/IAB8 recombinant described earlier. The minimal PTS was inserted into the modified Bluescript plasmid containing *frt* sequences flanking a unique BamHI site (see above), and then inserted into the BglII site of the C4PLZ/*gypsy*/IAB8 vector (see diagram below Figure 5A).

The *P* transformation vector shown in Figure 5C was prepared as follows. A 0.9 kb EcoRI-PstI PCR fragment (48,720–49,636) containing the *Abd-B*m promoter was inserted into the EcoRI-BamHI

sites of the pCaSper vector. A SpeI-BamHI fragment containing the 340 bp *gypsy* insulator DNA was inserted into the BamHI-SpeI sites of the Bluscript SK$^+$ vector. The BglII fragment of >PTS> was then cloned into the BamHI site. This vector was linearized by digestion with SmaI and PstI, and then ligated with a blunted EcoRI-PstI fragment containing the minimal IAB8 enhancer. A partial XbaI-PstI restriction fragment containing the *su(Hw)* insulator, PTS, *frt* sites, and IAB8 was cloned into the *Abd-B*/pCaSper transformation vector after digestion with XbaI and PstI.

P Transformation and In Situ Hybridization

P element transformation vectors containing *lacZ* and *white* reporter genes were introduced into the *Drosophila* germline by injecting *yw*^{67} embryos as described previously (Rubin and Spradling, 1982). Ten independent transformants were obtained for the recombinant *P* element shown in Figure 4, and four exhibited specific IAB7*–*white* interactions. A total of 36 independent lines was obtained for the recombinant *P* transformation vector shown in Figure 5A, and eight exhibited strong IAB8–*lacZ* interactions. One of the lines displayed strong expression of the distal *white* reporter gene (rather than the proximal *lacZ*). With the exception of this one line, IAB8 activity was blocked after removal of the PTS. Out of 12 lines obtained for the construct in Figure 5C, three displayed strong AE1–*Abd-B* promoter interactions, which are lost upon removal of the PTS.

In situ hybridizations were performed essentially as described by Zhou et al. (1999).

Fly Strains

Transgenic flies expressing the Flip recombinase were kindly provided by Gary Struhl and Steve Small (described in Wu et al., 1998). The *Abd-B* 3′ deficiency strain *Df(3R)S10/Dp(3;3)P5, Sb* was kindly provided by Ian Duncan (see Hopmann et al., 1995). The *iab-7*^{R73}*/TM6 Tb* was a kind gift of Shigeru Sakonju (see Hendrickson and Sakonju, 1995). *p302* (also known as *fs(3)5649/CxD*) was obtained from the Bloomington stock center (BL#P302).

Tissue Preparations

Adult abdominal cuticles were mounted for light microscopy as described by Duncan (1982).

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